AUTHOR(S): KIM, J., DE CASTRO, A., LENDOIRO, E., CRUZ-LANDEIRA, A., LOPEZ-RIVADULLA, M. and CONCHEIRO, M.

TITLE: Detection of in utero cannabis exposure by umbilical cord analysis.

YEAR: 2018

Publisher citation: KIM, J., DE CASTRO, A., LENDOIRO, E., CRUZ-LANDEIRA, A., LOPEZ-RIVADULLA, M. and CONCHEIRO, M. 2018. Detection of in utero cannabis exposure by umbilical cord analysis. Drug testing and analysis [online], 10(4), pages 636-643. Available from: https://doi.org/10.1002/dta.2307

OpenAIR citation: KIM, J., DE CASTRO, A., LENDOIRO, E., CRUZ-LANDEIRA, A., LOPEZ-RIVADULLA, M. and CONCHEIRO, M. 2018. Detection of in utero cannabis exposure by umbilical cord analysis. Drug testing and analysis, 10(4), pages 636-643. Held on OpenAIR [online]. Available from: https://openair.rgu.ac.uk

Full title: Detection of In Utero Cannabis Exposure by Umbilical Cord Analysis

Short title: Detection of In Utero Cannabis Exposure by Umbilical Cord Analysis

Jiyoung Kim[1], Ana de Castro[2], Elena Lendoiro[2,3], Angelines Cruz-Landeira[2], Manuel López-Rivadulla[2], Marta Concheiro[1,*]

[1] Department of Sciences, John Jay College of Criminal Justice, City University of New York, New York, USA

[2] Sección de Toxicología, Instituto de Ciencias Forenses, Universidad de Santiago de Compostela, Santiago de Compostela, Spain

[3] School of Pharmacy and Life Sciences, The Robert Gordon University, Aberdeen, United Kingdom

* Corresponding author:

Marta Concheiro, Ph.D.

Assistant Professor in Toxicology

Department of Sciences

John Jay College of Criminal Justice

City University of New York

524 West 59th Street – Rm 5.66.02

New York, NY 10019

Phone: 212-237-8492

Email: mconcheiro-guisan@jjay.cuny.edu

Funding

This project was funded by the Plan Nacional Sobre Drogas, Ministerio de Sanidad, Gobierno de España, grant 2016I005.

Abstract

According to 2014 National Survey on Drug Use and Health, 5.3% of pregnant women smoked marijuana in the past month. This prevalence is expected to increase as a growing number of states and countries are now considering legalization. Although umbilical cord is becoming a useful objective tool to detect in utero drug exposure, currently data about analytical methods and its utility to detect cannabis exposure are scarce. The objective of this work was to develop a method for the determination of Δ^9-tetrahydrocannabinol (THC), 11-hydroxyTHC (THC-OH), 11-nor-9-carboxy-THC (THCCOOH), 8-β-11-dihydroxyTHC (THC-diOH), THC and THCCOOH glucuronides, and cannabidiol (CBD) in umbilical cord by liquid chromatography-tandem mass spectrometry (LC-MS-MS) with dual ionization source. Umbilical cord samples (0.5g) were homogenized in methanol and extracted by solid phase extraction. Reversed-phase chromatographic separation was performed in 14 min, and two transitions per analyte were monitored in multiple reaction monitoring mode. Method validation included linearity (1-10 to 20-200ng/g), precision (4.1-23.4%), accuracy (87.5-111.4%), matrix effect (-54.8 to -5.8%), extraction efficiency (25-45.6%), limits of detection and quantification (1-10ng/g), and endogenous (n=5) or exogenous interferences (not detected). The method was applied to 13 authentic samples from cannabis-exposed newborns, which meconium samples had tested positive for cannabis. Twelve cord specimens tested positive for THCCOOH-Glucuronide (1.6-19.1ng/g). We developed and validated a specific and sensitive method for the simultaneous determination of THC, its metabolites, including THC and THCCOOH glucuronides, and CBD in umbilical cord samples by LC-MS/MS. The analysis of authentic samples showed the usefulness of umbilical cord to detect cannabis in utero exposure.

Keywords: umbilical cord, cannabis, THC, pregnancy, LC-MS/MS

1. Introduction

Cannabis is the one of the oldest and most widely used illicit drugs in the world. Due to the current increasingly permissive attitudes towards cannabis use and its legalization, the prevalence of cannabis has increased in recent years in the USA. According to National Survey on Drug Use and Health[1], 12.5 % of the population aged 12 or older reported past-year cannabis use in 2014. This prevalence was higher among men (16.92%) than women (10.11%), but both genders showed an increase (+4% men, +2.66% women) from 2002 to 2014 [2]. Focusing in the women group, prevalence for pregnant women past-year cannabis users also increased from 8.6 % in 2002 to 11.63% in 2014 [3].

Although the number of pregnant women using cannabis is rising, the adverse effects of fetal cannabis exposure are still less studied than the effects due to other illicit drugs [4]. Fetal cannabis exposure may produce impaired neurodevelopment, low birth weight, preterm labor, and more requirement to place the newborn in the intensive care unit [4–8]. However, there are no studies available that rigorously evaluate the impact of cannabis exposure on the developing fetus. The critical limitation in prior efforts has been inaccuracy of exposure assessment.

Self-report is the most economic and common method to monitor drug exposure during pregnancy, but maternal interview may be unreliable due to underreporting issues [9]. Analytical methods for the determination of drugs and metabolites in maternal and neonatal biological samples offer a more objective and reliable approach [10–12]. Neonatal specimens (meconium, hair and urine) directly reflect fetal exposure to drugs during pregnancy. Among these specimens, meconium is the most common one employed to detect fetal drug exposure during the 3rd trimester [12]. The main disadvantage of meconium is its collection; it may be delayed up to 5 days after birth, and if it passes before birth, drug testing cannot be performed.

Due to these reasons, umbilical cord has been suggested as an alternative specimen to detect in utero drug exposure. Umbilical cord is abundant, its collection is non-invasive and easy because it is considered as waste product [10,12]. Although its window of detection is still not established, several studies showed agreement between meconium and umbilical cord results, indicating a similar window of detection [13–15]. The current data available for cannabis in umbilical cord are scarce [14–16], and there is no information about the metabolite profile of THC in this biological samples, as well as at what concentrations these biomarkers are present.

Cannabis and metabolites have been determined in meconium [17–20], but umbilical cord methods are limited [21,22]. Chittama et al [21] developed a method for the determination of free THCCOOH in umbilical cord by immunoassay ELISA and liquid chromatography-time of flight mass spectrometry (LC-TOF). Montgomery et al [22] analyzed cannabinoids in umbilical cord by gas chromatography-mass spectrometry (GC-MS), but the authors did not indicate which cannabinoids were investigated, and no validation data were reported.

The aim of the present study was to develop and validate a sensitive and specific method for the simultaneous determination of delta-9-tetrahydrocannabinol (THC), cannabidiol (CBD) and five THC metabolites, 11-nor-9-carboxy-THC (THCCOOH), 11-hydroxy-THC (THC-OH), 8-β-11-dihydroxy-THC (THC-diOH), THCCOOH-glucuronide and THC-glucuronide, in umbilical cord by liquid chromatography tandem mass spectrometry (LC-MS/MS). The method was applied for the analysis of the authentic umbilical cord samples from newborns exposed to cannabis during pregnancy.

2. Materials and Methods

2.1. Chemicals and reagents

THC, THC-OH, THC-COOH and CBD at 1 mg/mL, and THCCOOH-glucuronide and the internal standards (IStd) THC-d_3, THC-OH-d_3 and THCCOOH-d_3 at 0.1 mg/mL in methanol were purchased from Cerilliant (Round Rock, TX, USA). THC-diOH and THC-diOH-d_6 at 0.1 mg/mL and THC-glucuronide at 0.01 mg/mL in methanol were from ElSohly Laboratories (Oxford, MS, USA). Solid phase extraction (SPE) mixed-mode cation exchange cartridges (Strata Drug X-C, 3 mL, 60 mg) were purchased from Phenomenex (Torrance, CA, USA). Formic acid was obtained from Pharmco-Aaper (Shelbyville, KY, USA). Methanol, acetonitrile, dichloromethane, and isopropanol were acquired from Fisher Scientific (Pittsburgh, PA, USA). Solvents for the extraction were high performance liquid chromatography (HPLC) grade and for the chromatographic instrument were liquid chromatography-mass spectrometry (LC-MS) grade.

2.2. Umbilical cord and meconium samples

Human blank umbilical cord samples were purchased from Lee BioSolutions (Maryland Heights, MO, USA), and were stored at -20 °C. Authentic samples were collected during a study, which aim was to investigate alternative matrices to detect in utero drug exposure [13]. Umbilical cord samples were collected at delivery in polypropylene containers and stored at -20 °C until analysis, and meconium samples were collected from newborn diapers up to 3 days after delivery in polypropylene containers and also stored at -20 °C until analysis. The participants were pregnant women who delivered at the University Hospitals of Santiago de Compostela and Vigo, Spain, from January 2012 to December 2015. The participants were informed about the study both in writing and orally before the delivery, and they gave written consent. The subjects were not paid for their participation. The study was approved by the Ethics Committee of the University of Santiago de Compostela, Spain.

2.3. Preparation of calibration and quality control umbilical cord samples

A working solution at 1 μg/mL containing all analytes except THC-glucuronide was prepared by dilution in methanol. Working solutions containing all analytes at 0.1 μg/mL and 0.01 μg/mL were prepared by dilution in methanol. Internal standard mixture at 0.1 μg/mL was prepared in methanol. All solutions were stored in amber vials at -20°C. Calibrators at 1, 2, 7, 10, 20, 50, 100 and 200 ng/g were prepared by the addition of proper volumes of 0.01, 0.1 or 1 μg/mL working solutions to 0.5 g blank umbilical cord samples. Low and high QC samples were prepared at 5 and 15 ng/g for THC-glucuronide, and at 15 and 150 ng/g for the other analytes.

2.4. Umbilical cord sample preparation

The umbilical cord sample was rinsed with ultra-pure water to remove maternal blood and biofluids. A segment of umbilical cord (0.5±0.02 g) was weighted and cut in small pieces. Three mL of methanol were added, and the samples were homogenized for 5 min using a tissue homogenizer (OMNI International, GA, USA) at speed 5.5. After centrifugation at 4960 rcf at 4°C for 15 min, the supernatants were transferred to new test tubes and evaporated at 55°C in a TurboVap LV evaporator (Biotage, Charlotte, NC, USA). The dried samples were reconstituted in 3 mL of a mixture of 0.1% formic acid in water and in acetonitrile (70:30, v/v), vortexed and centrifuged at 4960 rcf at 4°C for 5 min. The samples were loaded onto Strata X-C cartridges that were conditioned with 3 mL methanol and 3 mL deionized water. After loading the sample, the cartridges were washed with 2 mL of 1% formic acid in water and 2 mL of a mixture of 1% formic

acid in water and methanol (70:30, v/v). The cartridges were dried under vacuum for 10 min. Elution was performed by 3 mL of a mixture of dichloromethane and isopropanol (30:70, v/v), and the eluates were evaporated at 45°C in a TurboVap LV evaporator. The dried samples were reconstituted with 200 µL of a mixture of 0.1% formic acid in water and in acetonitrile (70:30, v/v), vortexed and transferred into screw top auto sampler vials.

2.5. Umbilical cord method LC-MS/MS Instrumentation

Ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) instrument LCMS-8030 from Shimadzu (Columbia, MD, USA) was employed. The Nexera UHPLC system consisted of a binary LC-30AD pump, online degassing unit DGU-20A and cooled autosampler SIL-30AC. The triple quadrupole mass spectrometer with Dual Ionization Source (DUIS) was operated in the positive ionization mode with the following parameters: spray voltage, 4.5 kV; corona pin voltage, 4.5 kV; desolvation line temperature 250°C; heat block temperature 400°C; nebulizing gas flow rate 2 L/min and drying gas flow rate 15 L/min. Precursor to product ion transition optimizations were performed by injection of individual analytes at 0.01 µg/mL in methanol without column. Each compound was monitored by 2 transitions in MRM, multiple reaction monitoring mode (Table 1). Chromatographic separation was conducted on a Kinetex F5 column, 100 × 2.1 mm, 1.7 µm (Phenomenex, Torrance, CA, USA) at 40°C. Mobile phase was 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B), at a flow rate of 0.5 mL/min, with the following gradient: initial composition of 30% B increased to 78.5% over 8.5 min, then increased to 98% over 0.2 min, held for 3 min, and then returned to initial composition over 0.2 min and held for 2.1 min for a total run time of 14 min.

2.6. Umbilical cord method validation

The Scientific Working Group for Forensic Toxicology (SWGTOX) guidelines [23] were employed for method validation and the following parameters were evaluated: linearity, limit of detection (LOD), limit of quantification (LOQ), carryover, precision, accuracy, matrix effect (ion suppression or enhancement), extraction efficiency, process efficiency, interferences and 24h-auto sampler stability.

Calibration model was evaluated with five different blank umbilical cord samples in five different days using five calibrator levels different from zero. Acceptable linearity was accomplished if the coefficient of determination (r^2) was ≥ 0.95 and the residuals were $< 20\%$. Limit of detection (LOD) and quantification (LOQ) were determined by fortifying five different umbilical cord samples at decreasing concentrations of compounds. Acceptance criteria for LOD involved signal-to noise > 3, retention time within ± 0.2 min from calibrators, and correct ion ratio qualifier to quantifier transition. LOQ met LOD criteria and had to quantify with acceptable precision (%CV < 20) and accuracy (80-120% of target concentration). Carryover was evaluated by injecting an extracted blank sample fortified with only IStd after an extracted sample fortified at 20 ng/g for THC-Glucuronide and 200 ng/g for other analytes (upper limit of quantification).

Precision and accuracy were evaluated at low and high QC concentrations, 5 ng/g and 15 ng/g for THC-Glucuronide, and 15 ng/g and 150 ng/g for others, in triplicate on five different days (n=15) using 5 different umbilical cord samples. Acceptable precision was achieved if the coefficient of variation (%CV) was less than 20%. Intra-assay precision was determined by the worst precision from the 5 different umbilical cord samples on 5 different days, and inter-assay precision considering all the replicates (n=15). Accuracy was represented as the percentage of the target concentration and was required to be within 80-120%.

For matrix effect, extraction efficiency and process efficiency evaluation, three sets of samples were prepared at low and high QC: blank samples fortified with analytes and IStd before (n=3) and after the extraction (n=6), and neat solutions (n=3). Matrix effect was evaluated by the comparison of the average peak area of samples fortified after extraction and neat solutions, (MeanAreaAfter/MeanAreaNeat - 1) x 100%. Process efficiency was calculated by comparing the average peak area of samples fortified before extraction and neat solutions, (MeanAreaBefore/MeanAreaNeat) x 100%. Extraction efficiency was calculated by the comparison of average peak areas in blank specimen fortified with analytes and IStd before and after extraction, (MeanAreaBefore/MeanAreaAfter) x 100%.

Endogenous interferences were analyzed in five different blank umbilical cord specimens fortified with only IStd. Exogenous interferences were evaluated injecting neat solutions at 200 ng/g of the following compounds: opiates (morphine, codeine, 6-monoacetylmorphine), amphetamines (amphetamine, methamphetamine, MDA, MDMA, MDEA), cocaine (benzoylecgonine, cocaine), methadone and benzodiazepines (alprazolam, clonazepam, flunitrazepam, diazepam). Autosampler stability was evaluated injecting low and high QCs stored at 10°C for 24 h in autosampler.

2.7. Meconium samples analysis

Meconium samples were analyzed by a previously published method [19]. Briefly, 0.25±0.02g meconium were homogenized in methanol and subjectred to cation exchange solid-phase extraction. The extracts were analyzed by LC-MS/MS Quattro Micro™ API ESCI triple quadrupole (Waters Corp., Milford, MA, USA). Chromatographic separation was performed on a Kinetiex C18 column 50x2.1mm, 2.6 µm from Phenomenex at 35°C with a gradient of 0.1% formic acid in water and acetonitrile at a flow rate of 0.3 mL/min, with a total run time of 10 min. Two transitions per compound were monitored in MRM positive mode. The method was fully validated, the linearity ranged from 4 to 400 ng/g for all analytes, except for THC glucuronide (10-400 ng/g).

3. Results

3.1. Method validation

The calibration model was linear, with $1/x^2$ weighting, non-forced through zero. The linearity range was from 1 to 20 ng/g for THC-Glucuronide, 1-200 ng/g for THCCOOH-Glucuronide, 7-200 ng/g for THCCOOH, CBD and THC, and 10-200 ng/g for THC-diOH and THC-OH. The coefficients of determination (r^2) of the calibration curves were ≥ 0.97 for all analytes, and residuals were less than 20%. The limits of detection and quantification ranged from 1 to 10 ng/g. Table 2 shows the results for the linearity, LOD and LOQ for each compound. Precision and accuracy for each analyte were measured at low and high QC concentrations. Figure 1 shows a umbilical cord sample fortified at the LOQ. Total precision ranged from 4.1 to 15.1%, intra-assay precision from 7.3 to 23.4%, and accuracy ranged from 87.5 to 111.4% (Table 3).

Extraction and process efficiency ranged from 25 to 45.6%, and 11.6 to 42.2%, respectively. Matrix effect (ion suppression) was from -54.8 to -5.8% with its variation being ≤

20% for all analytes, except for THC-glucuronide low QC (35.5%) and THCCOOH high QC (24.2%). Table 4 summarizes these results.

No carryover was observed in a blank sample fortified with only IStd when it was injected after a sample fortified at 20 ng/g for THC-Glucuronide and 200 ng/g for other analytes. No quantifiable peaks were detected from five different blank umbilical cords at the retention time of each analyte. In addition, there were no exogenous interferences from opiates (morphine, codeine, 6-monoacetylmorphine), amphetamines (amphetamine, methamphetamine, MDA, MDMA, MDEA), cocaine (benzoylecgonine, cocaine), methadone and benzodiazepines (alprazolam, clonazepam, flunitrazepam, diazepam). Autosampler stability was evaluated by reinjecting three low and high QCs stored at 10°C for 24 h in autosampler. No loss of any analyte was observed.

3.2. Authentic samples

The present method was applied to thirteen authentic umbilical cord samples (sample ID 1-13) from cannabis-exposed newborns, which meconium tested positive for THC and/or its metabolites, THC-OH, THC-diOH, THCCOOH, THC-glucuronide and/or THCCOOH-glucuronide, and for CBD. Six mothers denied cannabis use during pregnancy (sample ID 1, 4, 5, 8, 10 and 12), four admitted sporadic use (sample ID 3, 6, 9 and 11) and 2 daily use (sample ID 2 and 13). Umbilical cord authentic samples were analyzed in 3 different batches. Each batch included a negative control (blank umbilical cord fortified with the IStd mixture only), calibrators, and low and high QCs.

Twelve samples out of thirteen tested positive for cannabis in umbilical cord, showing a good agreement between umbilical cord and meconium (92.3% match). The predominant analytes in meconium samples were THCCOOH (n=12, 3.9 – 117.8 ng/g) and THC-diOH (n =11, 5.4 – 887.4 ng/g). Four samples tested positive for THCCOOH-Glucuronide (19.4 – 190.2 ng/g), three samples for THC (3 – 15.6 ng/g) and two for THC-OH (3.7 – 164.2 ng/g). Nine samples were positive for CBD (9.8 – 335.3 ng/g). In the case of umbilical cord, THCCOOH-glucuronide was the only metabolite detected in the twelve samples at concentrations below 20 ng/g (1.6 -19.1 ng/g). These results are shown in Figure 2 and Table 5.

4. Discussion

Meconium, neonatal hair and urine are commonly used for detection of in utero cannabis exposure. Due to the limitations of meconium, neonatal hair and urine, as difficulty of collection and sample availability, umbilical cord has been suggested as an alternative specimen [10,12]. The present method was developed and validated for the simultaneous analysis of THC and its metabolites THC-OH, THC-diOH, THCCOOH, THC-glucuronide and THCCOOH-glucuronide, and CBD in umbilical cord samples using LC-MS/MS.

Chittama et al. [21] developed a method for the screening of free THCCOOH in umbilical cord samples by ELISA and LC-TOF, and compared these methods' performance against results obtained by GC-MS, analyzed by an outside laboratory (USDTL, Des Plaines, IL, USA). The ELISA and LC-TOF method required 1 g of umbilical cord, and the LOQ was 0.1 ng/g (ELISA) and 1 ng/g (LC-TOF). Although our method's LOQ of THCOOH was higher (7 ng/g), the present method also confirms and quantifies THC, THC-OH, THC-diOH, THC-glucuronide, THCCOOH-glucuronide and CBD, in 0.5 g of umbilical cord, achieving LOQs 1 – 10 ng/g. During the validation, all parameters were within the range of the established criteria [23], except matrix effect

variability (CV) for THC-glucuronide low QC (35.5%) and THCCOOH high QC (24.2%). The use of the appropriate internal standard compensated for these effects, and they did not compromise other parameters such as precision, accuracy, LOD and LOQ. With regard to the chromatographic separation, we evaluated different reversed-phase stationary phases, C18, phenyl-hexyl and pentafluorophenyl (F5) phases, with mobile phase 0.1% formic acid in water and acetonitrile. The main difference among the different columns was the glucuronides peak shape and resolution between CBD and THC. The best results were obtained with the F5 phase.

Currently there are scarce data about the utility of umbilical cord to detect cannabis exposure during pregnancy [14,16,21], and there is no information about the predominant metabolites detected in this matrix. It is important to know this information because false negative results may occur from variations in the forms of THC metabolites present in a biological sample. Chittama et al. [21] reported free THCCOOH concentrations in umbilical cord by GC-MS from 0.06 to 6.09 ng/g (n=7). In twelve umbilical cord samples from newborns exposed to cannabis during pregnancy, we found only THCCOOH-Glucuronide at concentrations between 1.6 and 19.1 ng/g. We did not detect free THCCOOH, but our LOQ was 7 ng/g, higher than the concentrations previously reported for this metabolite (0.06 -6.09 ng/g) [21]. Glucuronide metabolites have been reported as the predominant analytes in umbilical cord for other drugs, such as buprenorphine [24] and morphine [13,25]. Umbilical cord consists in three blood vessels surrounded by Wharton's jelly. Wharton's jelly is a gelatinous substance mainly made up of mucopolysaccharides, which are highly polar and attack water [26]. This composition of the tissue may explain why polar metabolites such as glucuronides are accumulated in this matrix. In the present study, only one segment of the umbilical cord samples was analyzed, since homogeneous distribution of different types of analytes in this tissue were previously reported[24,27].

We analyzed thirteen umbilical cord samples from newborns, which meconium tested positive for THC, its metabolites, and/or CBD. Twelve out of thirteen were positive in umbilical cord, showing a 92.3% agreement; however, the metabolite profile was different in both matrices. In umbilical cord only THCCOOH-Glucuronide was detected, while in meconium free THCCOOH and THC-diOH were the predominant analytes, as previously reported [17,28,29]. Montgomery et al. [15] reported a 90.7% agreement between meconium and umbilical cord for cannabinoids detection; however, no information about target analytes and concentrations were reported. Colby et al. [14] also found a good agreement between umbilical cord and meconium (76%). Out of 217 paired samples, 27 were positive in meconium and umbilical cord, 36 positive samples in meconium and negative in cord, 17 positive samples in cord and negative in meconium, and 137 negative samples in both matrices. In that study, only THCCOOH was analyzed in meconium (confirmation cut-off 5 ng/g) and in umbilical cord (screening cut-off 150 ng/g). Although the authors stated that the Chittama et al. [21] method was employed for the analysis of umbilical cord samples (cut-off of 0.1 ng/g in ELISA, 1 ng/g in LC-TOF), a surprisingly high THCCOOH cut-off in umbilical cord (150 ng/g) was reported. Palmer et al. [16] reported non-difference between meconium and umbilical cord in overall detection of cannabis exposure during pregnancy. In this study, meconium and umbilical cord samples were not paired, and a comparison of the number of cases detected with these matrices was performed (7.7% in meconium, 9% in umbilical cord). Umbilical cord samples were screened by ELISA for free THCCOOH [21], but no confirmation was performed.

A limitation of this study could be that only umbilical cord samples, which meconium tested positive to cannabis, were analyzed. Therefore, we did not evaluate if umbilical cord

samples could have tested positive and meconium negative. Based on the obtained results, this situation may be marginal, but further research should be performed. Another observation it is the higher concentrations observed in meconium compared to umbilical cord, and the different metabolite pattern in both biological samples. The different composition of the umbilical cord tissue and meconium, the physicochemical properties of the compounds, fetal metabolism, maternal dosing and timing may influence different accumulation in meconium and umbilical cord. The inclusion of additional metabolites, such as THC-OH glucuronide, which has been reported in meconium samples [17,28,29], may improve the umbilical cord detection. Currently, there are no data available about the clinical significance of these analytical results in umbilical cord samples. Gray et al. [30] compared neonatal growth outcomes in cannabis-exposed and non-exposed neonates, as determined by meconium analysis, and found reductions in gestational age, birth weight, head circumference, and length, yet most neonates were within the expected range for weight, head circumference, and length. More data are necessary to confirm THCCOOH-glucuronide as predominant metabolite in umbilical cord, to evaluate additional THC biomarkers in this biological sample, and to establish its window of detection and its clinical interpretation.

5. Conclusion

We developed and validated a specific and sensitive method for the determination of THC, its metabolites, including THC and THCCOOH glucuronides, and CBD in umbilical cord samples by LC-MS/MS. The analysis of authentic samples demonstrated the value of umbilical cord in detecting in utero cannabis exposure, and that THCCOOH-Glucuronide was the only THC metabolite detected in these samples.

Acknowledgements

This project was funded by the Plan Nacional Sobre Drogas, Ministerio de Sanidad, Gobierno de España, grant 2016I005. E. Lendoiro would like to thank the Consellería de Cultura, Educación e Ordenación Universitaria, Xunta de Galicia, for her postdoctoral contract (ED481B2016/103-0).

The authors would like to thank Elmer Morales and Vicent Blandino from John Jay College for their technical assistance during sample preparation.

Conflict of Interest

None of the authors have any conflict of interest to disclose.

References

1. Center for Behavioral Health Statistics and Quality. Key Substance Use and Mental Health Indicators in the United States: Results from the 2015 National Survey on Drug Use and Health. *HHS Publ*. 2016; No. SMA 16-4984, NSDUH Series H-51. Retrieved from http://www.samhsa.gov/data/

2. Carliner H, Mauro PM, Brown QL, et al. The widening gender gap in marijuana use prevalence in the U.S. during a period of economic change, 2002–2014. *Drug Alcohol Depend*. 2017;170:51-58.

3. Brown QL, Sarvet AL, Shmulewitz D, et al. Trends in Marijuana Use Among Pregnant and Nonpregnant Reproductive-Aged Women, 2002-2014. *JAMA*. 2016;72(12):1235-1242.

4. Gunn JKL, Rosales CB, Center KE, et al. Prenatal exposure to cannabis and maternal and child health outcomes: a systematic review and meta-analysis. *BMJ Open*. 2016;6(4):e009986.

5. Hayatbakhsh MR, Flenady VJ, Gibbons KS, et al. Birth Outcomes Associated With Cannabis Use Before and During Pregnancy. *Pediatr Res*. 2012;71(2):215-219.

6. Merritt TA, Wilkinson B, Chervenak C. Maternal Use of Marijuana during Pregnancy and Lactation : Implications for Infant and Child Development and Their Well-Being. *Acad J Pediatr Neonatol*. 2016;2(1):1-8.

7. Fergusson D, Horwood L. Maternal use of cannabis and pregnancy outcome. *BJOG An Int J Obstet Gynecol*. 2002;109:21-27.

8. Goldschmidt L, Richardson GA, Willford J, Day NL. Prenatal Marijuana Exposure and Intelligence Test Performance at Age 6. *J Am Acad Child Adolesc Psychiatry*. 2008;47(3):254-263.

9. Garg M, Garrison L, Leeman L, et al. Validity of self-reported drug use information among pregnant women. 2016;20(1):41-47.

10. Gray T, Huestis M. Bioanalytical procedures for monitoring in utero drug exposure. *Anal Bioanal Chem*. 2007;388(7):1455-1465.

11. Lozano J, Garcia-Algar O, Vall O, De La Torre R, Scaravelli G, Pichini S. Biological Matrices for the Evaluation of In Utero Exposure to Drugs of Abuse. *Ther Drug Monit*. 2007;29(6):711-734.

12. Concheiro-Guisan A, Concheiro M. Bioanalysis during pregnancy: Recent advances and novel sampling strategies. *Bioanalysis*. 2014;6(23):3133-3153.

13. Concheiro M, Lendoiro E, de Castro A, et al. Bioanalysis for cocaine, opiates, methadone, and amphetamines exposure detection during pregnancy. *Drug Test Anal*. 2017;9(6):898-904.

14. Colby JM. Comparison of umbilical cord tissue and meconium for the confirmation of in utero drug exposure. *Clin Biochem*. 2017;50(13-14):784-790.

15. Montgomery D, Plate C, Alder SC, Jones M, Jones J, Christensen RD. Testing for fetal exposure to illicit drugs using umbilical cord tissue vs meconium. *J Perinatol*. 2006;26(1):11-14.

16. Palmer KL, Wood KE, Krasowski MD. Evaluating a switch from meconium to umbilical cord tissue for newborn drug testing: A retrospective study at an academic medical center. *Clin Biochem*. 2017;50(6):255-261.

17. Gray TR, Barnes AJ, Huestis M a. Effect of hydrolysis on identifying prenatal cannabis exposure. *Anal Bioanal Chem Chem*. 2010;397(6):2335-2347.

18. Marchei E, Pellegrini M, Pacifici R, et al. Quantification of D9-tetrahydrocannabinol and its Major Metabolites in Meconium by Gas Chromatographic-mass Spectrometric Assay: Assay Validation and Preliminary Results of the "'Meconium Project.'" *Ther Drug Monit*. 2006;28:700-706.

19. Prego-Meleiro P, Lendoiro E, Concheiro M, Cruz A, López-Rivadulla M, de Castro A. Development and validation of a liquid chromatography tandem mass spectrometry method for the determination of cannabinoids and phase I and II metabolites in meconium. *J Chromatogr A*. 2017;1497:118-126.

20. Ristimaa J, Gergov M, Pelander A, Halmesmäki E, Ojanperä I. Broad-spectrum drug screening of meconium by liquid chromatography with tandem mass spectrometry and time-of-flight mass spectrometry. *Anal Bioanal Chem*. 2010;398(2):925-935.

21. Chittamma A, Marin SJ, Williams JA, Clark C, McMillin GA. Detection of in utero marijuana exposure by GC-MS, ultra-sensitive ELISA and LC-TOF-MS using umbilical cord tissue. *J Anal Toxicol*. 2013;37(7):391-394.

22. Montgomery DP, Plate CA, Jones M, et al. Using umbilical cord tissue to detect fetal exposure to illicit drugs: a multicentered study in Utah and New Jersey. *J Perinatol*. 2008;28(11):750-753.

23. Scientific working group for forensic toxicology. Scientific working group for forensic toxicology (SWGTOX) standard practices for method validation in forensic toxicology. *J Anal Toxicol*. 2013;37(7):452-474.

24. Concheiro M, Jones HE, Johnson RE, Choo R, Shakleya DM, Huestis MA. Umbilical cord monitoring of in utero drug exposure to buprenorphine and correlation with maternal dose and neonatal outcomes. *J Anal Toxicol*. 2010;34:498-505.

25. Concheiro M, González-Colmenero E, Lendoiro E, et al. Alternative Matrices for Cocaine, Heroin , and Methadone In Utero Drug Exposure Detection. *Ther Drug Monit*. 2013;35:502-509.

26. Di Naro E, Ghezzi F, Raio L, Franchi M, D'Addario V. Umbilical cord morphology and pregnancy outcome. *Eur J Obstet Gynecol Reprod Biol*. 2001;96(2):150-157.

27. de Castro A, Jones HE, Johnson RE, Gray TR, Shakleya DM, Huestis MA. Methadone, Cocaine, Opiates, and Metabolite Disposition in Umbilical Cord and Correlations to Maternal Methadone Dose and Neonatal Outcomes. *Ther Drug Monit*. 2011;33(4):443-452.

28. Feng S, ElSohly M a, Salamone S, Salem MY. Simultaneous analysis of delta9-THC and its major metabolites in urine, plasma, and meconium by GC-MS using an immunoaffinity extraction procedure. *J Anal Toxicol*. 2000;24(6):395-402.

29. Elsohly MA, Feng S. delta9-THC metabolites in meconium: identification of 11-oh-delta9-THC, 8beta,11-dioh-delta9-THC, and 11-nor-delta9-thc-9-cooh as major metabolites of delta9-thc. *J Anal Toxicol*. 1998;22:329-335.

30. Gray TR, Eiden RD, Leonard KE, Connors GJ, Shisler S, Huestis MA. Identifying prenatal cannabis exposure and effects of concurrent tobacco exposure on neonatal growth. *Clin Chem*. 2010;56(9):1442-1450.

Table 1. Liquid chromatography mass spectrometry parameters for delta-9-tetrahydrocannabinol (THC), cannabidiol (CBD) and five THC metabolites, 11-nor-9-carboxy-THC (THCCOOH), 11-hydroxy-THC (THC-OH), 8-β-11-dihydroxy-THC (THC-diOH), THCCOOH-glucuronide and THC-glucuronide in umbilical cord. The quantifier transition is underlined.

Analyte	MRM Transition	Collision energy(v)	Ion ratio Qualifier/Quantifier (calibrators' average)	RT (min)	IStd
THC-diOH	347 > 311.2 347 > 173	-17 -31	0.2	3.1	THC-diOH-d_6
THC-diOH-d_6	353.1 > 317.4 353.1 > 178.9	-16 -25		3.2	
THCCOOH-glucuronide	521.1 > 345.1 521.1 > 326.9	-15 -18	0.2	3.4	THCCOOH-glucuronide-d_3
THCCOOH-glucuronide-d_3	524.1 > 348.2 524.1 > 330.1	-15 -21		3.5	
THC-glucuronide	491.4 > 315.1 491.4 > 192.9	-17 -39	0.2	4	THCCOOH-glucuronide-d_3
THC-OH	331.4 > 201.2 331.4 > 133.1	-27 -25	1	4.6	THC-OH-d_3
THC-OH-d_3	334.1 > 196.1 334.1 > 123	-30 -40		4.6	
THCCOOH	345 > 299.2 345 > 193	-21 -28	0.5	4.8	THCCOOH-d_3
THCCOOH-d_3	348.1 > 330. 348.1 > 302	-17 -23		4.8	
CBD	315.4 > 192.9 315.4 > 135	-23 -20	0.5	5.9	THC-d_3
THC	315.1 > 193.1 315.1 > 122.9	-23 -38	1.1	6.6	THC-d_3
THC-d_3	318.1 > 195.9 318.1 > 122.8	-27 -39		6.6	

Table 2. Linearity parameters, linear range, and LOD/LOQ for delta-9-tetrahydrocannabinol (THC), cannabidiol (CBD) and five THC metabolites, 11-nor-9-carboxy-THC (THCCOOH), 11-hydroxy-THC (THC-OH), 8-β-11-dihydroxy-THC (THC-diOH), THCCOOH-glucuronide) and THC-glucuronide in umbilical cord (n=5).

Analyte	LOD/LOQ (ng/g)	Linearity Range (ng/g)	Intercept±SD	Slope±SD	r^2±SD
THC-diOH	10	10-200	0.1018±0.080	0.55±0.05	0.975±0.009
THCCOOH-glucuronide	1	1-200	0.1376±0.111	4.61±1.37	0.986±0.006
THC-glucuronide	1	1-20	-0.1075±0.079	5.64±1.62	0.983±0.014
THC-OH	10	10-200	-0.0237±0.110	0.35±0.08	0.979±0.003
THCCOOH	7	7-200	0.0002±0.021	0.33±0.07	0.976±0.009
CBD	7	7-200	0.2192±0.195	0.85±0.21	0.978±0.013
THC	7	7-200	0.0593±0.108	0.82±0.07	0.979±0.007

Table 3. Precision and accuracy (n=15) for delta-9-tetrahydrocannabinol (THC), cannabidiol (CBD) and five THC metabolites, 11-nor-9-carboxy-THC (THCCOOH), 11-hydroxy-THC (THC-OH), 8-β-11-dihydroxy-THC (THC-diOH), THCCOOH-glucuronide) and THC-glucuronide in umbilical cord at low and high QC (low QC at 5 ng/g and high QC at 15 ng/g for THC-Glucuronide, and low QC at 15 ng/g and high QC at 150 ng/g for other compounds).

Analyte	Intra-day Precision (CV, %)		Inter-day Precision (CV, %)		Total Accuracy (%)	
	Low QC	High QC	Low QC	High QC	Low QC	High QC
THC-diOH	11.1	7.3	9.3	4.1	107.4	111.4
THCCOOH-Glucuronide	10.0	11.4	10.3	11.2	104.0	106.7
THC-Glucuronide	10.5	16.2	9.5	12.4	103.1	104.5
THC-OH	11.8	7.8	12.8	15.1	100.4	97.6
THCCOOH	13.4	8.0	11.1	12.6	103.9	105.2
CBD	17.2	17.6	14.5	14.3	87.5	97.4
THC	23.4	7.9	12.5	4.6	100.4	108.2

Table 4. Matrix Effect (ion suppression or enhancement), Extraction Efficiency, and Process Efficiency for delta-9-tetrahydrocannabinol (THC), cannabidiol (CBD) and five THC metabolites, 11-nor-9-carboxy-THC (THCCOOH), 11-hydroxy-THC (THC-OH), 8-β-11-dihydroxy-THC (THC-diOH), THCCOOH-glucuronide) and THC-glucuronide in umbilical cord at low and high QC (low QC at 5 ng/g and high QC at 15 ng/g for THC-Glucuronide, and low QC at 15 ng/g and high QC at 150 ng/g for other compounds).

Analyte	Matrix Effect (CV, %) (n=6)		Extraction Efficiency (n=3)		Process Efficiency (n=3)	
	Low QC	High QC	Low QC	High QC	Low QC	High QC
THC-diOH	-44.2 (6.1)	-29.7 (3.4)	29.7	39.6	16.6	27.8
THCCOOH-glucuronide	-21.4 (7.7)	-5.8 (8)	38.3	44.8	30.1	42.2
THC-glucuronide	-43.5 (35.5)	-28.8 (14.1)	25.0	30.2	14.1	21.5
THC-OH	-54.8 (7.7)	-47.1 (12.6)	25.6	37.8	11.6	20.0
THCCOOH	-50.6 (11.6)	-44.4 (24.2)	28.1	39.5	13.9	22.0
CBD	-26.4 (11.7)	-33.2 (7.1)	31.2	36.0	22.9	24.0
THC	-41.6 (20)	-50.3 (17.2)	44.4	45.6	25.9	22.0

Table 5. Authentic matched meconium and umbilical cord samples from thirteen newborns exposed to cannabis during pregnancy. THC, delta-9-tetrahydrocanninol; CDB, cannabidiol; THC-COOH, 11-nor-9-carboxy-THC; THC-OH, 11-hydroxy-THC; THC-diOH, 8-β-11-dihydroxy-THC; THCCOOH-glucuronide, 11-nor-9-carboxy-THC– glucuronide. ND, not detected.

Sample ID	Meconium (ng/g)						Umbilical Cord (ng/g)
	THCCOOH	THC-diOH	THCCOOH-glucuronide	THC	THC-OH	CBD	THCCOOH-Gluc
1	3.9	21.4	ND	ND	3.7	ND	ND
2	15.8	90	ND	ND	ND	ND	12.3
3	14.6	85	ND	ND	ND	9.5	1.6
4	48.4	887.4	ND	ND	164.2	ND	6.8
5	9.3	18.1	19.4	ND	ND	18.5	16.3
6	23.4	ND	ND	ND	ND	31.4	2.2
7*	45	53.2	190.2	5.6	ND	112.1	19.1
8	19.7	30.8	ND	ND	ND	56.5	4.3
9	117.8	5.4	ND	15.6	ND	335.3	3.6
10	6.2	88.2	ND	3	ND	30.8	3.2
11	44.4	ND	67.4	ND	ND	74.6	14.6
12	8.4	47.6	111.8	ND	ND	19.7	5.1
13	ND	43.2	ND	ND	ND	ND	2.8

*Note: Sample ID 7 meconium results were previously reported in reference[19], and it corresponds with sample 1910/2015.

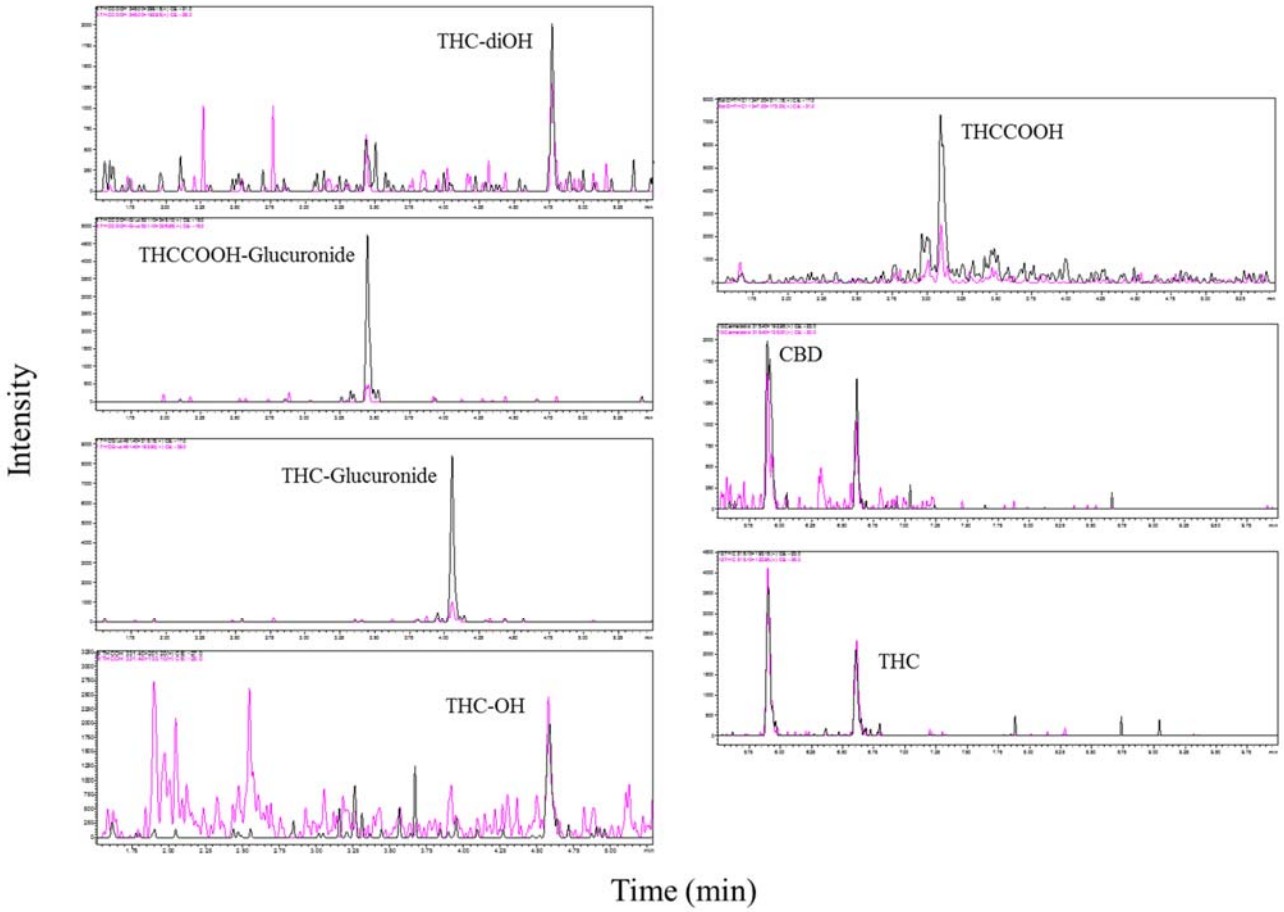

Figure1. MRM chromatograms of the quantifier (black line) and qualifier (pink line) transitions in blank umbilical cord fortified at the limit of quantification, 1 ng/g for THCCOOH-Glucuronide and THC-Glucuronide, 7 ng/g for THCCOOH, THC and CBD, and 10 ng/g for THC-OH and THC-diOH.

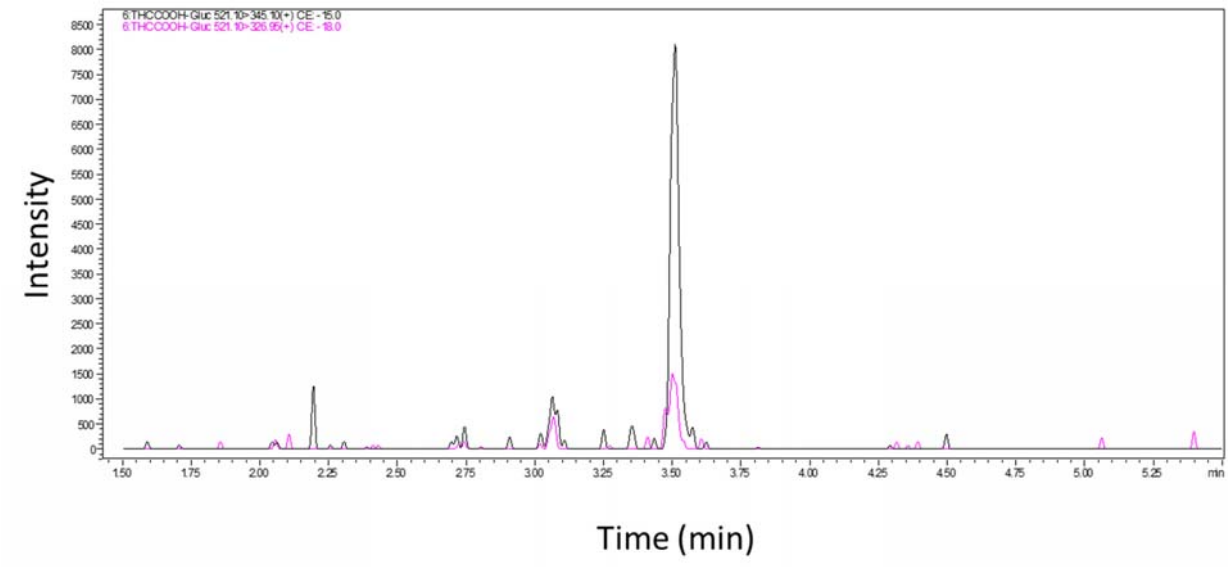

Figure 2. MRM chromatograms of the quantifier (black line) and qualifier (pink line) transitions in an authentic umbilical cord sample (sample ID 8) positive for THCCOOH-Glucuronide at 4.3 ng/g.